BIOASSAY, CARCINOGENESIS and TISSUE CULTURE

NO. 338-A

Cf. #338

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

SUCCESSOR TO THE
TOBACCO INDUSTRY RESEARCH COMMITTEE
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NEW YORK, N. Y. 10017

Activated on 10/1/62 and renewed annually thru October 1, 1966. Preceded by #29B, #212 & #224 - 1955 - 1962 incl. #456 is concurrent on a five year plan.

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Application For Research Grant

Date: August 18th, 1967

1. Name of Investigator: Freddy Homburger, M.D.

2. Title: President and Director

3. Institution & Address: Bio-Research Institute, Inc.
9 Commercial Avenue
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4. Project or Subject: BIOASSAY OF TOBACCO SMOKE CONDENSATES AND RELATED PROBLEMS

5. Detailed Plan of Procedure (Use additional pages if more space is required.)

INTRODUCTION

It is realized that our current grant from CTR has been made for one year without further commitment in order to enable us to conclude a five-year program initiated late in 1962.

The present application is to request the Council to consider support for another five-year program, based upon our past performance summarized in the progress report.

The three major projects for which we seek long-term support are the following:

1. Acceleration of growth of chemically induced tumors for the purpose of developing rapid carcinogen testing methods.

2. Systematic study of inhibitors of chemical carcinogenesis with the aim to neutralize alleged carcinogens contained in cigarette smoke condensates.

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Bioassay of Tobacco Smoke Condensates and Related Problems

3. Skin-painting studies in mice to measure under comparable conditions the decline of carcinogenicity and of co-carcinogenicity for mouse skin that appears to have occurred since 1960 in cigarette smoke condensate.

Each of these projects is based on many years of experience in the particular field and clearly promises to yield valuable fundamental scientific knowledge and will contribute to the technology necessary to formulate cigarettes that will produce smoke condensates incapable of producing cancers when painted on the skin of mice.

1) Acceleration of Carcinogen Testing

There are efforts underway in many laboratories to develop rapid screening procedures for the detection of carcinogenic substances. These range from in vitro tissue culture work to the use of neonatal mice, newts and other species. The destruction of sebaceous glands and the increased lethality of U.V. light for paramecia and other biological phenomena have been correlated with carcinogenic potency. However, the most reliable carcinogenesis test would still be the production of tumors in a mammalian species in a sufficiently short time to make it practical to detect even weak cancer-causing chemicals.

Our studies on the transfer of multiple pooled carcinogen injection sites represent the first significant step in this direction. We believe that it will be possible to obtain even shorter times of latency than so far possible by extracting from the initial carcinogen-injection or -application sites large numbers of transformed cells for transfer into fresh hosts.

Initial experiments on this subject are already underway. The subcutaneous injection sites of C57BL/6 mice are excised after 3 to 5 weeks of contact with carcinogen (benzo[rst]pentaphene is being used as standard carcinogen) and after mechanical dispersion by means of Snell's cytosieve, the cell suspensions are centrifugated in Ficoll®, a neutral high molecular dextran-like polysaccharide of low osmotic pressure. The cells are thereby separated according to their specific gravity and the various cell layers, some of which will contain concentrated amounts of malignant cells, are injected into fresh hosts. By this method, it is possible to inject into a single mouse many times the number of transformed cells coming from numerous induction sites. Based on the studies of several authors using transplanted tumors and confirmed by our own work, the larger the number of transferred malignant cells, the more rapid the growth of tumors. It is believed that it may be possible to get a 100% tumor yield 2 weeks after transfer into new hosts or 5 to 7 weeks after the carcinogen was first injected.

Bioassay of Tobacco Smoke Condensates and Related Problems

In the mouse, the first cytologically malignant cells are found in carcinogen injection sites in the subcutaneous tissue five weeks after injection of carcinogen. By transfer of pooled multiple injection sites, we were observing changes in from 2 to 3 weeks after carcinogen injection but not earlier. Thus, the shortest theoretically possible test period would be 4 weeks.

In hamsters, on the other hand, Nettleship and Smith (Proc. Soc. Exp. Biol. Med. 74:800-802, 1950) showed morphologically transformed fibroblasts 24 hours after injection of methylcholanthrene. Hence, it would appear that the subcutaneous tissue of hamsters would lend itself even better than that of the mouse to carcinogen testing. The times of latency in hamsters for subcutaneously induced tumors is the same as in mice. It is likely (assuming that Nettleship's observations can be confirmed) that transfer of multiple injection sites in hamsters and even more so, transfer of transformed cell concentrates, will produce palpable tumors in a shorter period than in mice. Such transfers could be made 1 or 2 days after injection of carcinogen and the shortest possible test period would be 2 to 3 weeks.

In addition, chromosome studies are readily feasible in hamsters and tumor cells transferred from females into males could be identified as belonging to the original female host and hence as induced by the carcinogen injected into the first (female) host.

We propose to establish during the next 2 to 3 years a tissue culture laboratory under the direction (either part time or later on full time) of Dr. Janis Gabliks, currently Associate Professor of Cell Biology at Massachusetts Institute of Technology, and to carry out the above described studies.

In addition, it will then become possible to extend the work of Berwald and Sachs on transformation of hamster fibroblasts in vitro and to transfer large numbers of cells exposed to carcinogens in vitro back into hamster cheek pouches. In this way, it may be possible to obtain tumor growth even more rapidly than is possible with in vivo systems alone.

We are quite confident that these techniques applied to subcutaneous tissue, epidermis and lung tissue of mice and hamsters will make such procedures as mouse-skin painting obsolete and replace them by carcinogen tests lasting less than two months and having as endpoints histologically demonstrable malignant tumors, the truly neoplastic nature of which can be ascertained by serial transplantation.

While these studies will initially be done with chemical carcinogens (strong, weak and intermediate), we shall soon be able to apply them as

well to tobacco smoke condensates, since the condensate producing machine of Bio-Research Consultants, originally scheduled for this spring, will be in operation later this summer and condensate will be available in adequate amounts.

2) Systematic Study of Inhibitors of Chemical Carcinogenesis

While Falk and Kotin showed that reduction derivatives of polycyclic hydrocarbons inhibit the carcinogenic effect of these hydrocarbons, we found that oxidative derivatives also have this effect.

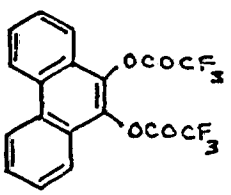
The quinones appear to be a group of special interest because they possess chemotherapeutic (in contrast to merely carcinogenesis inhibiting) effects. The hypothesis that hydrogen peroxide, which may form from quinones, is the cytotoxic agent is susceptible to test by synthetizing the aza derivatives corresponding to the quinones which, if the peroxide hypothesis were correct, would not possess the chemotherapeutic activity of the quinones. Conversely, the di-quinone should be more active.

It is the purpose of a systematic study of derivatives of benzo [rst]pentaphene, of other polycyclic hydrocarbons and of terpenes (such as limonene) and their derivatives to find those compounds that are most active in counteracting the carcinogenic effects of polycyclic hydrocarbons and that are themselves least carcinogenic. Such compounds could be used eventually to neutralize the carcinogenic effects (as tested by our own new rapid methods and by skin painting) of cigarette smoke condensates.

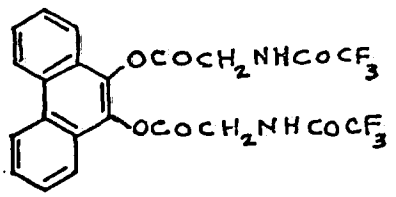
In order to render these compounds more volatile and better suited for use as adjuvants in the tobacco blends, it is suggested to prepare and test the trifluoroacetyl derivatives of some hydroquinones and their glycyl esters, which are known to be precursors of the cytotoxic quinones in vivo.

These compounds are represented by the following examples:

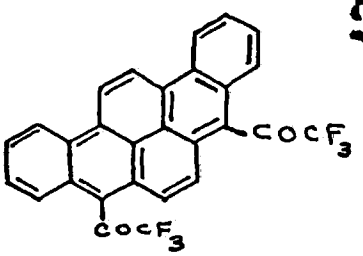
1. 9,10-Phenanthrene hydroquinone di(trifluoroacetate), IV
2. 9,10-Phenanthrene hydroquinone-bis-trifluoroacetylglycyl ester, V
3. 3,4,9,10-dibenzpyrene-5,8-di(trifluoroacetoxy), VI



IV



V



VI

This approach is based on methods used in gas chromatography for the volatilization of amino acids (see, for example, Cruickshank and Sheehan, Anal. Chem. 36:1191, 1964). The trifluoroacetate group is known to split very easily, and the trifluoroacetic acid and its derivatives are completely non-toxic and physiologically inert (see "Toxic Aliphatic Fluorine Compounds" by F. L. M. Pattison, Elsevier Pub. Co., 1959, pp. 20;27;62).

3) Skin-Painting Studies in Mice

Skin painting in mice has been used since Croninger, Graham and Wynder's early work, for lack of a better method, as a measure of carcinogenicity of smoke condensates.

There are indications that present day cigarettes may be less carcinogenic and less co-carcinogenic in terms of mouse skin response than those originally studied by Wynder, Kensler and ourselves. Such observations on reduced carcinogenicity of cigarette smoke condensates have been published by Wynder and by Bock. However, neither of these authors was in a position to prepare at the time of his latest study condensates from cigarettes used in his earlier (more carcinogenic) experiments. We have some 20,000 cigarettes made in 1960 of cigar tobacco which have been preserved and which could serve as a control for a comparison between mouse carcinogenicity of condensates of 1960 and of 1967.

The evidence which suggests such an experiment is shown in Table I, summarizing our own mouse-skin painting experiments using condensates from various unfiltered cigarettes done in 1960, 1963, 64, 65 and 66.

There is a strong suggestion here that the mouse-skin carcinogenicity has declined and, even more striking, that co-carcinogenicity has practically disappeared. While the cigarettes used were the same brand in most of these studies, the source of the condensates, the machines used for smoking and the handling of the condensates were different. For these reasons, the results shown here are only suggestive and not conclusive.

We are convinced, however, that a repetition of our earlier study sponsored by CTR in 1960 to 61 would yield similar and conclusive evidence if indeed the composition of present day cigarettes has changed. We are planning the experiment summarized in Table II.

Condensates could be prepared by Bio-Research Consultants in an identical manner for all cigarettes smoked. Condensates would be diluted with equal parts of acetone as in our previous studies.

Bioassay of Tobacco Smoke Condensates and Related Problems

By use of the ATC cigarettes (which we obtained from Dr. Hockett and understand to be made of the cigar tobacco used in our original 1960 experiment), we could obtain a check on the reproducibility of the first (1960) experiment and thereby determine that CAF_1 mice respond today in a manner similar to 1960. We could by this device obtain a comparison with the Millerton mice used by Wynder in most of his published studies. Inclusion of the two most widely smoked non-filter cigarettes of today is logical.

The use of primed animals having received 400γ of benzpyrene as initiator measures the co-carcinogenicity of the condensates, and the use of croton oil as a promotor yields early information on the carcinogenicity of the condensates.

This is an elaborate, complex and protracted (two years) experiment. It will provide conclusive evidence not obtainable by any other means and might well demonstrate the absence of co-carcinogenicity and greatly attenuated carcinogenicity of present day cigarettes.

In view of our latest skin-painting studies (Table I) such an outcome may be predicted as likely.

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Table I

SUMMARY OF SOME RECENT MOUSE SKIN PAINTING EXPERIMENTS

Date of beginning of project	1961* -62	Jan. 1963	May 1964	July 1964	April 1965	Dec. 1965		Feb. 1966		August 1966	
Mice	CAF-1/Jax ♀♀	Millerton Swiss ♀♀	Charles River Swiss ♀♀	CAF-1/Jax ♀♀	Millerton Swiss ♀♀	CAF-1/Jax ♀♀					Millerton Swiss ♀♀
Primed with benzo[a]pyrene†	-	-	+	-	+	+	-	-	-	-	-	+
Cigarette condensate applied:												
Per cent tar	50%	50%	50%	18.6%	50%	50%		18.6%	50%	18.6%	50%	
Per cent water	-	-	-	15.4%	-	-		15.4%	-	-		
No. of mice per group at start	100	200	45	200	150	100	50	50	50	50	100	60
Papillomas after:												
30 weeks				0.5%								
40 "			56%		14%							
42 "	1%	10%	58%			8%	0%	0	2%	0		
57 "	9%			1%								
66 "	20%	39%		6%				0	7%	2%		
76 "	32%	60%		12%		21%	7%					

* Published J. Nat. Cancer Inst. 31:1445, 1963.

† 400μg per mouse.

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Table II

PROPOSED EXPERIMENT FOR CTR MOUSE SKIN PAINTING

Group No.	Strain of Mice	No. Mice per Group	Treatment of Mice		
			Primer* (mg per mouse)	Cigarette Smoke Condensate Derived from**	Croton Oil
1	CAF/1	100	-	ATC†	-
2	"	100	-	Brand A	-
3	"	100	-	Brand B	-
4	"	100	-	none#	-
5	Millerton Swiss	100	-	ATC†	-
6	"	100	-	Brand B	-
7	"	100	-	none#	-
8	CAF/1	50	0.4	ATC†	-
9	"	50	0.4	Brand B	-
10	"	50	0.4	-	0.75
11	"	50	0.4	none#	-
12	"	50	-	-	0.75
Total		950			

* Benzo[a]pyrene
** All cigarettes of regular length (70 mm)
† All-tobacco cigarettes
# Acetone control

6. Budget Plan: *

Based on current level of endeavor.

a.	Salaries	34,760.
b.	Expendable Supplies	5,300.
c.	Other Expenses	3,085.
d.	Permanent Equipment	6,000.
e.	Overhead (15% of a, b, c)	6,472.
	Total	55,617.

* 2/1/68-1/31/69

7. Anticipated Duration of Work: Five years

8. Facilities and Staff Available: The same personnel as in the past will remain active in this work. In addition, a new group is being developed for studies on tissue cultures and hamster oncology in our new Cummington Street annex. It is anticipated that with addition of increased staff in the new division for work with hamsters, some of the mouse work will be reduced so that during each of the next five years, the budget for this project will remain approximately the same.

9. Additional Requirements: Condensates for the carcinogen inhibition and carcinogen acceleration studies will be provided by Bio-Research Consultants free of charge for labor costs only because their smoking machine was developed under CTR contract. In the case of skin-painting studies where large amounts of condensate are required, this will have to be purchased by CTR at the cost incurred by necessary addition of technical personnel for the production of these large amounts of condensate. However, it is anticipated that the new condensate machine will be so much more efficient than existing smoking machines that this cost will be small.

10. Additional Information (Including relation of work to other projects and other sources of support)

In the past, it has been possible to share some personnel with our carcinogenesis studies carried out under National Institutes of Health, National Cancer Institute Research Grant No. CA-04869. Since this grant has been discontinued, this is no longer possible. Thus, support for these studies by CTR is assuming added importance for us. Even with it (at the requested rate), we must reduce our total effort in the carcinogenesis field. Without CTR support, we should have drastically to reduce our efforts in this field in which we have worked since 1948 and for which we have developed a uniquely competent team.

Signature Frederick Hamburger

Director of Project

Business Officer of the Institution